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A model wheat cultivar for transformation to improve resistance to Fusarium Head Blight

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Abstract Fusarium head blight (FHB), caused primarily by *Fusarium graminearum*, is a major disease problem in wheat (*Triticum aestivum*). Genetic engineering holds significant potential to enhance FHB resistance in wheat. Due to the requirement of screening for FHB resistance on flowers at anthesis, the number of screens carried out in a year is limited. Our objective was to evaluate the feasibility of using the rapid-maturing dwarf wheat cultivar Apogee as an alternative genotype for transgenic FHB resistance research. Our transformation efficiency (number of transgenic plants/number of embryos) for Apogee was 1.33%. Apogee was also found to exhibit high FHB susceptibility and reached anthesis within 4 weeks. Interestingly, microsatellite marker haplotype analysis of the chromosome 3BS FHB resistant quantitative trait locus (QTL) region indicated that this region maybe deleted in Apogee. Our results indicate that Apogee is particularly well suited for accelerating transgenic FHB resistance research and transgenic wheat research in general.

Keywords Apogee · *Fusarium graminearum* · Fusarium head blight · Transgenic wheat · *Triticum aestivum*

Abbreviations FHB: Fusarium head blight · 2,4-D: 2,4-dichlorophenoxyacetic acid · GUS: β -glucuronidase · QTL: quantitative trait locus

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Introduction

Fusarium head blight (FHB; scab), caused primarily by the fungus *Fusarium graminearum*, (teleomorph *Gibberella zeae* (Schwein.) Petch) is a devastating disease of wheat and other small grains. In the Upper Midwest of the United States alone, FHB caused an estimated one billion dollars in losses in 1993 (McMullen et al. 1997), and total economic losses due to FHB in the U.S. between 1993 and 2001 have been estimated at nearly 8 billion dollars (Nganje et al. 2004). Many major wheat production regions in North America, South America, Asia, and Europe face the threat of serious epidemics of FHB (Parry et al. 1995). The fungus infects spikes during and shortly after anthesis and results in reduced grain yield. Additionally, the fungus produces trichothecene mycotoxins that result in reduced grain quality (Bai and Shaner 1994; Sutton 1982; Tuite et al. 1990). Genetic resistance, management practices and fungicides are all used for partial control of the disease. Genetic resistance is the most promising and environmentally friendly approach and many groups are developing wheat lines with enhanced FHB resistance.

Large-scale breeding efforts are being pursued to incorporate genetic resistance to FHB (e.g., Rudd et al. 2001). While progress has been made in improving FHB resistance through breeding, the resistance so far identified is only partial and quantitative in nature (e.g., Kolb et al. 2001). Thus, alternative strategies for improving FHB resistance offer an attractive complement to traditional breeding. Genetic engineering is one such approach that holds promise. Wheat transformation methodologies were developed in the 1990's (Vasil et al. 1992; Weeks et al. 1993; Becker et al. 1994; Nehra et al. 1994), and many researchers are now examining the potential of transforming wheat to enhance FHB resistance. Chen et al. (1999) developed transgenic wheat expressing a rice thaumatin-like protein1 and found that this line delayed development of FHB symptoms. Anand et al. (2003) produced transgenic wheat lines expressing chitinase and β -1,3-glucanase genes that provided moderate resistance to FHB in greenhouse

trials. In addition, transgenic wheat expressing the *F. sporotrichioides* *Tri101* gene, which encodes a protein that detoxifies the trichothecene mycotoxins produced by *F. graminearum*, conferred partial protection against the spread of FHB in infected spikes (Okubara et al. 2002). Thus, preliminary efforts to employ genetic engineering to improve FHB resistance are encouraging.

One significant obstacle to developing FHB-resistant wheat by genetic engineering is that only adult plants can be screened for resistance. This limits the number of generations that can be screened in a year. Currently, the wheat transformation community largely uses the cultivar Bobwhite for transformation (e.g., Campbell et al. 2000; Hu et al. 2003; Weeks et al. 1993; Cheng et al. 1997; Srivastava et al. 1999), however a wide variety of wheat genotypes have been transformed (e.g., Rasco-Gaunt et al. 2001). Bobwhite is widely used for transformation work because of its high regeneration and transformation efficiency when compared to other cultivars. Fortuitously for FHB research, Bobwhite is also moderately susceptible to this disease, which allows the identification of improved resistance in transformants. However, Bobwhite matures slowly, and this amplifies the temporal obstacle of disease evaluations limited to adult plants. To accelerate transgenic FHB resistance studies, it would be desirable to have a FHB-susceptible wheat genotype that can be readily transformed and that matures more rapidly than Bobwhite, thus allowing more generations of wheat transformants to be cycled each year. Finally, identifying a transformation amenable wheat genotype that is rapidly maturing would accelerate all wheat transgenic research.

“USU-Apogee” (Apogee) is a dwarf hard red spring wheat cultivar developed at Utah State University in cooperation with NASA (Bugbee et al. 1997). Apogee has been found to exhibit adequate bread-making qualities (Veillard and Kokini 2001; Bugbee et al. 1997). In addition, Apogee exhibits rapid generation turnover; under continuous light at 25°C, heads are reported to emerge 23 days after seedling emergence (Bugbee et al. 1997). In this paper, we describe several attributes of Apogee that make it an alternative model cultivar for accelerating efforts to improve FHB resistance in wheat by transformation and for general transgenic wheat research.

Materials and methods

Plant materials

The spring wheat cultivars Apogee, Wheaton, Norm, Sumai 3, Bobwhite, and Alsen were used for the experiments.

Wheat transformation

The plasmid pAHC25 (Christensen et al. 1992) containing the *uidA* gene under the control of the maize ubiquitin promoter and the *bar* gene under the control of the same promoter was kindly donated by Dr. Peter Quail

(Plant Gene Expression Center, University of California, Berkeley). The *uidA* gene encodes β -glucuronidase, (GUS), and the *bar* gene encodes the enzyme phosphinothricin acetyltransferase, which confers resistance to the herbicide Bialaphos.

Spring wheat plants (cv. Apogee) were grown in growth chambers under a 16 h photoperiod at 20°C during the day and 18°C at night. Transformation was carried out as described by Campbell et al. (2000) with modifications. Ten to fourteen days after anthesis, immature seeds were collected and surface sterilized. Embryos were excised and placed on callus induction medium, scutellum side up, for 4 days, in the dark, at 24°C. Callus induction medium consisted of Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog 1962) supplemented with 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D); 2.2 mg/l 4-amino-3,5,6-trichloropicolinic acid (picloram) and 40 g/l maltose, at pH 5.8. Callusing embryos were placed on osmoticum medium for 4 h, in the dark, at 24°C. Osmoticum media consisted of callus induction media supplemented with 1.25 M raffinose and 1.25 M mannitol. Embryos were then bombarded with 0.6 μ m gold particles (BioRad, Hercules, CA), coated with 5 μ g DNA from plasmid pAHC25. Precipitation of the DNA onto the gold particles and bombardments using a Biolistic PDS-1000/He Particle Delivery System (Dupont, Wilmington, DE) with disposable components supplied by BioRad (BioRad, Hercules, CA) were carried out according to Weeks et al. (1993) with modifications. Following bombardment, embryos were placed overnight in the dark at 24°C. The next day, the embryos were moved onto callus induction media supplemented with 5 mg/l Bialaphos (Meiji Seika Kaisha, Ltd., Japan) and placed in the dark at 24°C for 2 weeks. Embryogenic callus were then divided into smaller pieces and placed on shoot regeneration media (supplemented with 5 mg/l Bialaphos) for 2 weeks under a 16 h photoperiod at 24°C. Care was taken to label callus pieces derived from the same embryo lineage. Shoot regeneration medium consisted of MS salts and vitamins supplemented with 0.25 mg/l 2,4-D and 40 g/l maltose, pH 5.8. Pieces of callus developing shoots were then placed onto root regeneration media (supplemented with 5 mg/l Bialaphos) for 2 weeks, under a 16 h photoperiod, at 24°C. Root regeneration medium consisted of MS salts and vitamins, supplemented with 40 g/l maltose. Plantlets, which were producing shoots and roots were then placed on root regeneration medium (supplemented with 5 mg/l Bialaphos) for 4 weeks under a 16 h photoperiod at 24°C. Small sections of leaf were then assayed histochemically for GUS activity using X-gluc (Labs Scientific, Inc., Livingston, NJ) as the substrate according to the procedure described by Jefferson et al. (1987). Plantlets expressing the *uidA* gene were transplanted to soil, and grown in growth chambers under the conditions described above.

DNA gel blot analysis

DNA isolation, gel blot analysis, hybridization and washes were performed according to de la Peña et al. (1999). The

uidA gene probe was derived from a PCR amplified product. The forward and reverse primers used for the *uidA* PCR amplification were 5'-GACAAGGCACTAGCGGGACT-3', and 5'-ATTGACGCAGGTGATCGGAC-3', respectively.

FHB susceptibility of Apogee

Plants were grown four to a pot (Magnum 6 in. squares, Belden Plastics, Roseville, MN) containing Metromix 200 (Scotts Co., Marysville, OH) in a greenhouse. Plants were fertilized with Osmocote (14-14-14 N-P-K, Scotts Co., Marysville, OH), and stagger-planted so that inoculations could be conducted on the same day for all genotypes. At anthesis, one floret near the middle of each selected spike was inoculated with 10 μ l of a *F. graminearum* conidial suspension consisting of an admixture of approximately 15 separate isolates at a concentration of 50,000 macroconidia ml^{-1} (kindly provided by Dr. Ruth Dill-Macky, Department of Plant Pathology, University of Minnesota). Inoculated plants were placed in a dew chamber for 72 h to promote infection, and then were removed from the dew chamber and placed back in the greenhouse to allow disease symptoms to develop. Disease symptoms were assessed visually at 21 days after inoculation, by scoring both incidence of infection, and severity (percentage of spikelets on an inoculated spike that exhibited disease damage). The wheat cultivar Wheaton was used as a susceptible check, while the moderately-resistant cultivar Alsen was used as a resistant check.

Growth characteristics of Apogee vs. Bobwhite

Apogee and Bobwhite were grown in both growth chamber and greenhouse studies to compare growth parameters that were deemed relevant to the use of Apogee as a model genotype for transformation and subsequent FHB screening. Growth chamber studies involved growing Apogee and Bobwhite in pots as described above. Three plants per genotype were planted in each pot, with four pots per genotype. Plants were fertilized with one teaspoon of Osmocote fertilizer per pot. Plants were placed in a growth chamber set at 20°C with 16 h day/8 h night. Information recorded included days to anthesis, plant height, spike length, and spikelets per spike. The growth chamber experiment was repeated twice. Greenhouse studies were conducted in a manner similar to those described for the growth chamber experiment. Four separate plantings were undertaken on different dates and in two separate greenhouses during the month of September, to obtain average growth characteristics in a month during which greenhouse plantings for FHB evaluations commonly occur.

Molecular marker analysis of Apogee

The marker haplotype of Apogee on the short arm of chromosome 3B (3BS) in the region harboring a major QTL

conferring FHB resistance, *Qfhs.ndsu-3BS* (Waldron et al. 1999), was determined using a set of microsatellite markers that span the QTL region (Song et al. 2005). DNA was extracted from Apogee, Sumai 3, Norm, and Wheaton using a modified small-scale version of the protocol detailed in Riede and Anderson (1996). Microsatellite markers from the genome region of interest (gwm389, barc75, gwm533, barc133, barc147, gwm493, and barc102) were used to amplify the appropriate markers from the wheat genotypes by PCR. Final reactions contained 1X reaction buffer, 1.5 mM MgCl_2 , 0.2 mM dNTPs, 0.1 μ M each primer, and 0.025 U μl^{-1} Taq DNA polymerase. Cycling consisted of an initial 3 min denaturing cycle at 94°C, followed by 35 cycles of amplification consisting of 94°C (1 min), 1 min primer annealing at the temperature recommended for each primer, and a 72°C extension for 2 min. The PCR products were separated by electrophoresis on vertical denaturing 7% polyacrylamide gels, and then were visualized by silver staining to compare the allele composition for these markers across the genotypes.

Results and discussion

Production and characterization of transgenic Apogee

Six-hundred immature embryos from the cultivar Apogee were bombarded with the pAHC25 plasmid. pAHC25 contains the *Bar* and *uidA* genes driven by the maize ubiquitin promoter for selection on Bialaphos and visualization of GUS activity, respectively. Selection of transgenic tissue cultures and plants was conducted on Bialaphos-containing media (see materials and methods). To identify transformants, leaf tissue from plants growing on Bialaphos was histochemically stained for GUS activity. We identified eight GUS-expressing T_0 plants.

To verify that the eight plants were transgenic, to determine the GUS expression patterns in aerial tissues, and to determine if each of the events were independent, we characterized each event for GUS expression and transgene integration in the genome. To identify transgenic T_1 seed, endosperm from T_1 seed from each of the eight T_0 transformants was histochemically stained for GUS activity (Fig. 1). GUS-positive T_1 seed from each event were planted in the greenhouse and tested for GUS activity in leaves and reproductive structures. Moderate to strong GUS expression was found in these tissues from all of the transgenic events (Fig. 1). Bract tissue was also observed to exhibit moderate to high levels of GUS expression (data not shown). Additionally, DNA gel blot analysis was performed on the eight transformants. Genomic DNA from the T_1 generation from each of the lines was digested with *HindIII*, blotted and hybridized with a probe from the *uidA* reporter gene. The probe did not hybridize to the DNA from nontransformed Apogee but each transgenic Apogee line exhibited a unique banding pattern, indicating that each line was an independent transformation event (Fig. 2).

Our GUS expression and DNA gel blot results show that the wheat cv. Apogee can be transformed with

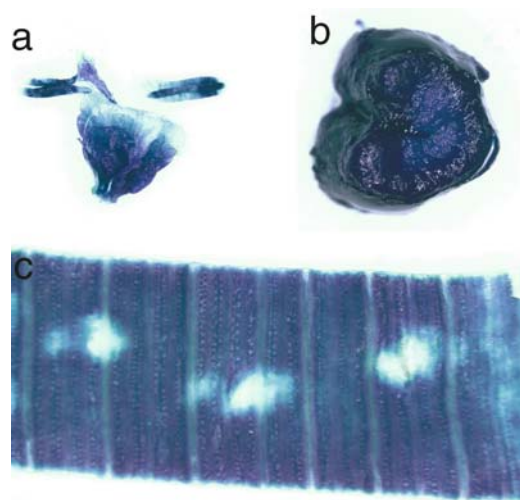


Fig. 1 a–c Histochemical GUS-staining of tissues from Apogee T₁ plants. **a** Ovule and anthers. **b** Endosperm; **c** Leaf tissue. All transgenic events generated exhibited moderate to strong GUS expression in all tissues examined

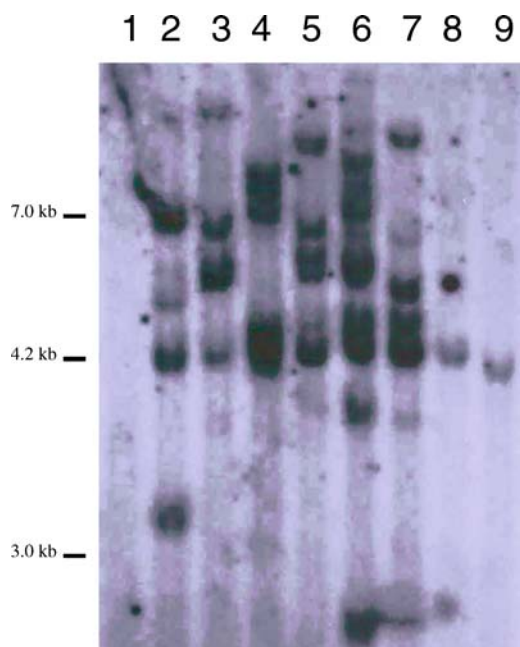


Fig. 2 DNA gel blot of eight T₁ generation Apogee transformants. Lane 1 is Apogee and lanes 2–9 are the eight transgenic events. All transformants display unique banding patterns showing that they are independent transgenic events

an efficiency of approximately 1.33% (eight transgenic events/600 embryos). In addition, GUS expression driven by the ubiquitin promoter can be detected in aerial tissues of Apogee, similar to our experience with Bobwhite (G.J. Muehlbauer, unpublished results). In our hands, the transformation efficiency in Apogee is similar to our 1–3% efficiency with the wheat cv. Bobwhite (G.J. Muehlbauer, unpublished results). These results show that Apogee is a useful alternative wheat genotype for transformation research.

FHB susceptibility of Apogee

Apogee exhibited susceptibility that was comparable to that of Wheaton, a susceptible cultivar commonly used in FHB screening. Point inoculation of Apogee (20 spikes) with an admixture of isolates of *F. graminearum* resulted in 100% incidence and a mean disease severity (percentage of florets on a spike exhibiting blight symptoms) of 63% (SE = 6%). By comparison, the susceptible check Wheaton (21 spikes) also exhibited 100% disease incidence and a mean disease severity of 61% (SE = 7%), while the resistant check Alsen (21 spikes) exhibited a disease incidence of 100% but a disease severity of just 13% (SE = 2%). The FHB susceptibility of Apogee has been confirmed by other research groups (H.C. Kistler and R. Dill-Macky, personal communication). Thus, Apogee transformants with increased FHB resistance should be readily identifiable. Indeed, improved FHB resistance in transformants may be detected more readily in Apogee, based on a small comparative FHB evaluation of Apogee and Bobwhite that indicated greater susceptibility of Apogee to FHB than Bobwhite (data not shown). An additional observation is that Apogee tolerates dew chamber treatment better than other wheats, based upon the observation that it does not exhibit noticeable yellowing of leaves after the dew chamber incubations.

Growth characteristics of Apogee vs. Bobwhite

Growth characteristics of Apogee and Bobwhite under both growth chamber and greenhouse growing conditions are shown in Table 1 and Fig. 3. Apogee reached anthesis nearly 40% faster than Bobwhite (28 vs. 45 days) in the growth chamber. The average difference under greenhouse conditions was 15 days, but both genotypes took longer to reach anthesis (39 days for Apogee vs. 54 days for Bobwhite). Further, the flowering habit of Apogee is highly consistent in growth chamber conditions, with nearly all plants in a given experiment flowering within a day of each other. In contrast, Bobwhite plants flowered between 41 and 49 days in one experiment, and between 45 and 49 days in the other experiment. The desirable dwarf habit of

Table 1 Morphological characteristics of Apogee and Bobwhite grown in growth chamber and greenhouse conditions

Cultivar	Anthesis (d)	Plant height (cm)	Spike length (cm)	Spikelets/spike
Growth chamber				
Apogee	28 (2.2)	33 (2.6)	8.3 (0.4)	15 (0.1)
Bobwhite	45 (1.8)	54 (2.1)	10.3 (0.4)	19 (0.1)
Greenhouse				
Apogee	39 (1.7)	45 (2.7)	6.9 (0.3)	13 (0.5)
Bobwhite	54 (2.2)	65 (3.5)	9.5 (0.3)	19 (0.6)

Values for the growth chamber represent the means of two separate experiments planted on different dates, with 11 to 12 observations per genotype per experiment. Values for greenhouse evaluations are the means of four separate experiments planted on different dates and/or in different greenhouses. Standard errors are given in parentheses



Fig. 3 Growth comparison of Apogee and Bobwhite grown in a growth chamber. Apogee is on the left-hand side of the picture. The scale bar is equivalent to 10 cm

Apogee is revealed in the comparison of heights of Apogee plants at maturity relative to Bobwhite (33 vs. 54 cm and 45 vs. 65 cm, in growth chamber and greenhouse conditions respectively). Fortunately, while Apogee plants possess a dwarf stature, its spike characteristics such as spike length and the number of spikelets per spike are still acceptable for FHB evaluations (Table 1). Indeed, both the spike lengths and spikelets per spike for Apogee were 80% that of Bobwhite in the growth chamber experiments, while these values were approximately 70% of Bobwhite across the four greenhouse experiments (Table 1).

Apogee was developed as a rapid-maturing wheat cultivar for possible growth in space research work. Our work, comparing the growth of Apogee versus Bobwhite highlights the advantages that Apogee presents as a model cultivar for wheat genetic engineering research. The significantly faster flowering times of Apogee translate both into faster disease evaluations and generation turnover times. In fact, we have been able to obtain fully mature seeds from growth chamber-grown Apogee plants in two months, without imposing any additional conditions to accelerate the maturation process. This generation turnover is far faster than that achieved with Bobwhite. Thus, in a calendar year, it should be possible to advance as many as six complete generations

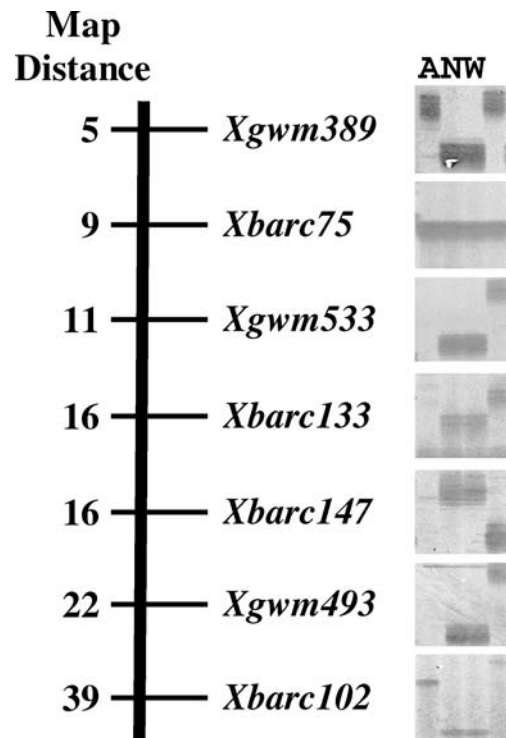


Fig. 4 Chromosome arm 3BS microsatellite marker amplification in Apogee and other wheat genotypes. Marker locus orders and approximate map distances are based on the report of Song et al. (2005). A, Apogee; N, Norm; W, Wheaton; S, Sumai 3

of Apogee plants under growth chamber conditions. This process could be accelerated even further by stopping watering of plants earlier or by reducing nutrient supply. The short plant stature of Apogee is also desirable. There is no need for staking plants, thus saving labor costs. The rigid stems of Apogee ensure that there is no lodging during the moving and manipulating of plants for disease evaluations.

Molecular marker analysis of Apogee

To initiate an understanding of the genetic basis for the FHB susceptibility in Apogee, we determined the microsatellite haplotype in Apogee at the chromosome 3BS type II FHB resistance QTL (*Qfhs.ndsu-3BS*; Waldron et al. 1999; Anderson et al. 2001). Surprisingly, primer pairs for the microsatellite markers gwm533, barc133, barc147, and gwm493 failed to amplify a product in Apogee (Fig. 4). The pattern of amplification, or lack thereof, was not random. Based on the recently published wheat genetic map, the microsatellite loci barc75 and barc102 flank the gwm533, barc133, barc147, and gwm493 microsatellite loci (Song et al. 2005). The barc75 and barc102 microsatellite markers exhibited amplified products in Apogee, delimiting the extent of null microsatellite alleles in this region from approximately 10 cm to a maximum of approximately 30 cm. The discovery of apparent null alleles for the region of chromosome arm 3BS harboring a major FHB resistance QTL is suggestive of a deletion of this chromosome region. We

are not aware of any other FHB wheat cultivars with this marker haplotype. If this is a deletion it will presumably have eliminated the locus at which *Qfhs.ndsu-3BS* QTL resides, underlying the high degree of FHB susceptibility in this cultivar.

We have demonstrated that the transformation efficiency of Apogee is equal to that of Bobwhite in our laboratory. This, coupled with the fact that Apogee is susceptible to FHB, reaches anthesis significantly faster than Bobwhite, in both greenhouse and growth chamber conditions, and possesses a more petite stature than Bobwhite that permits more dense planting and requires no staking, highlights the usefulness of Apogee for accelerating transgenic wheat FHB resistance research. Also, Apogee has been shown to exhibit adequate bread-making qualities (Veillard and Kokini 2001; Bugbee et al. 1997), indicating that it is a useful genotype for crossing into elite hard red spring wheats. In addition to the applicability to FHB research, our results demonstrate the utility of Apogee to accelerate all wheat transgenic research.

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